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PRINCIPAL INVESTIGATOR: Virginia A. Spencer
J. R. Davie, Ph.D.

CONTRACTING ORGANIZATION: University of Manitoba
Winnipeg, Manitoba, Canada R3E 0W3

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6. AUTHOR(S)
Virginia A. Spencer
J. R. Davie, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Manitoba
Winnipeg, Manitoba, Canada R3E 0W3

Vspence@cc.umanitoba.ca

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13. Abstract *(Maximum 200 Words) (abstract should contain no proprietary or confidential information)*
Estrogen affects both the cytoskeletal and chromatin structure of breast cancer cells. The treatment of breast cancer cells with 10 nM estradiol causes the estrogen receptor to recruit histone acetyltransferases to the promoter of the estrogen-responsive PS2 gene within 60 min, while exposure to estrogen for 72 h increases the levels of DNA-associated cytokeratins. To determine the effect of estrogen on regions downstream of estrogen-responsive promoters, quantitative PCR was performed on DNA isolated from chromatin immunoprecipitations using anti-acetylated H3 and H4 antibodies. Treatment with 10 nM estradiol induced a transient state of H3 and H4 acetylation along the PS2 promoter, exon 2, and exon 3, that was maximal after 60-120 min. After three hours, this hyperacetylation decreased to levels above those observed along the PS2 gene in untreated MCF-7 cells. To determine how estrogen influences DNA-associated cytokeratins, the levels of total, intermediate filament-assembled and DNA-associated cytokeratins were determined by Western Blotting and two dimension gel electrophoresis. The level of DNA-associated cytokeratins increased after 2 h of 10 nM estradiol treatment, while the levels of total and filament cytokeratins remained the same. This suggests that early estrogen exposure (i.e. 2 h) promotes interactions between cytokeratins and DNA.

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Introduction

Breast cancer proliferation is induced by estrogen, a hormone that induces the estrogen receptor to recruit histone acetyltransferases to the promoters of estrogen-responsive genes [1]. Upon recruitment, these enzymes acetylate the core histones H2A, H2B, H3 and H4, proteins that compose the nucleosome [2]. Acetylation of the core histones disrupts interactions between histones on adjacent chromatin fibers and causes chromatin to unravel; thereby allowing transcription factors access to their DNA-binding site [2]. The treatment of breast cancer cells with the anti-estrogen, ICI inhibits breast cancer development by causing the recruitment of histone deacetylases to the promoter of estrogen responsive genes [1;3]. These enzymes deacetylate core histones [2]. One of the goals of my research is to determine if estrogen and ICI affect the structure of chromatin along the promoter and exon regions of the PS2 gene, rather than just at the promoter alone.

The shape of a cell is governed by a dynamic tissue matrix system that links together three-dimensional skeletal networks from the nuclear matrix, cytoskeleton and extracellular matrix [4;5]. The NM binds to DNA regions along the chromatin fiber referred to as matrix attachment regions [6], organizing DNA into loop domains [2]. In addition to affecting the structure of chromatin, long term (72 h) exposure to estrogen increases the levels of cytoskeletal proteins such as cytokeratins [7;8]. Such proteins play an important role in the transduction of mechanical and chemical signals to the nucleus [9], and, therefore, are important in the development of breast cancer. In a previous study, exposure to estrogen for 72 h increased the levels of total and DNA-associated cytokeratins 8, 18 and 19 [7]. Whether estrogen alters DNA organization by increasing the contacts between cytokeratins and DNA or by simply increasing the levels of cytokeratins associated with DNA remains to be determined. The second goal of my research is to determine if the treatment of breast cancer cells with estrogen causes immediate (within 1-2 h) changes in the levels of DNA-associated, intermediate filament-assembled and/or total cytokeratins.

Body

Task 1: To determine the effect of estrogen and anti-estrogen ligand on histone H3 and H4 acetylation within the gene promoter and coding region of the pS2 estrogen-responsive gene.

Method:

MCF-7 cells were grown in estrogen-deplete conditions for 72 h, and then treated with 10 nM estradiol for 0-3 h. The cells were then treated with 1% formaldehyde to cross-link any pre-existing protein-DNA complexes, lysed, and sonicated to fragment the DNA to an average size of 300-400 bp. The lysate was subjected to chromatin immunoprecipitation using antibodies to di-acetylated H3 and penta-acetylated H4 (Upstate Biotechnology, NY) [1]. The DNA associated with acetylated H3 and H4 was isolated and analyzed

by quantitative PCR using primers designed to amplify a 315 bp, 239 bp, and 196 bp region from the PS2 promoter, exon 2 and exon 3 regions, respectively.

Results:

Analysis of DNA immunoprecipitated with acetylated H3 and acetylated H4 antibodies by PCR revealed that the levels of acetylated H3 and H4 along the PS2 promoter, exon 2 and exon 3 regions began to increase as early as 30 min following 10 nM estradiol treatment (Figures 1 and 2 of Appendix). The acetylation of H3 was maximal along the three PS2 regions 60 min following estradiol treatment, and decreased when the cells were incubated for another 2 h to levels still greater than those observed in untreated cells (Figure 1 of Appendix). The level of acetylated H4 also appeared maximal along the PS2 promoter and exon 2 regions after 60 min exposure to 10 nM estradiol, and subsequently decreased along the promoter when the cells were incubated for another hour in the presence of estradiol. However, no further decrease in acetylated H4 was observed along the promoter in the third hour of estradiol treatment. As well, exon 2 displayed a constant level of H4 hyperacetylation following exposure to estradiol for 1 to 3 h. Exon 3 became maximally acetylated after 120 min exposure to estradiol, and then displayed a sharp decrease in acetylated H4 in the third hour of treatment.

Future Work:

Future studies will determine the effect of ICI on the acetylation of H3 and H4 along the PS2 gene. As well, I will determine if the transient histone acetylation we observed along the PS2 gene is dependent on the presence of transcription. To accomplish this, I will treat MCF-7 human breast cancer cells with different transcription inhibitors (e.g. α-amanitin, DRB) in the presence of estradiol and determine how these agents affect estrogen-induced histone acetylation along the PS2 gene.

Task 2: To determine if the effect of estradiol on the re-establishment of DNA-associated cytoskeletal intermediate filament and microfilament arrays occurs simultaneously or before down-regulation of p21 expression (i.e. before 8 hours exposure to 10 nM estradiol).

Method:

MCF-7 cells were grown in estrogen-deplete conditions for 72 h, and then treated with 10 nM estradiol for 0-2 h. The isolation of cytokeratins and actin cross-linked to DNA was performed as previously described [7]. In brief, MCF-7 cells were cross-linked with 1 mM cisplatin for 2 h, suspended in lysis buffer (5M urea, 2M NaCl, 2M guanidine hydrochloride), and the DNA-protein complexes isolated by hydroxyapatite chromatography. The DNA-cross-linked proteins were then analysed either by two-dimensional gel electrophoresis followed by silver staining or by Western Blotting using an antibody to

cytokeratins 8 and 18 (Biodesign International, ME) and β-actin (Sigma, ON). To determine if the levels of total or intermediate filament-assembled cytokeratins as well as DNA-associated cytokeratins changed in response to estradiol treatment, intermediate filament assembled cytokeratins were isolated as previously described [7] and analysed by two-dimensional gel electrophoresis. As well, total intermediate filament proteins were analysed from cell lysates.

Results:

Two dimensional gel electrophoresis of cisplatin DNA-cross-linked proteins isolated from MCF-7 cells treated with 10 nM estradiol for 0-2 h revealed an increase in the levels of DNA-associated cytokeratins after 1 h treatment with estradiol, followed by a further increase after 2 h of treatment (Figure 3A in Appendix). However, in a repeat study, no significant difference in DNA-associated cytokeratin levels could be observed by two dimensional gel electrophoresis over the 2 h estradiol treatment (Figure 3B in Appendix). Therefore, to improve the level of detection of cytokeratins within each sample, Western Blot analysis using an antibody to cytokeratins 8, 18 and 19 was performed on DNA cross-linked protein samples isolated from subsequent experiments (Figure 3C and D and Appendix). The results of these Western Blot analyses showed an increase in cytokeratins 8 and 18 approximately 2 h after treatment with 10 nM estradiol, while the level of cytokeratin 19 did not increase (Figure 3 in Appendix). Two-dimensional patterns of total and intermediate filament-assembled proteins did not display any significant changes in the levels of cytokeratins after exposure to estradiol for 2 h (Figure 4 in Appendix).

Future Work:

Experiments confirming the levels of total, intermediate filament-assembled, and DNA-associated cytokeratins after 2 h treatment with 10 nM estradiol need to be performed by Western blot analysis. The effect of estradiol on total and DNA-associated actin filaments also needs to be determined.

Key Research Accomplishments:

From Task I:

- Treatment of MCF-7 cells with 10 nM estradiol induces a transient state of H3 and H4 acetylation along the PS2 promoter, exon 2, and exon 3 gene regions, that is maximal after 60-120 min exposure to estradiol. After three hours exposure to estradiol, this hyperacetylation decreases to levels above those observed along the PS2 gene in untreated MCF-7 cells.

From Task 2:

- Treatment of MCF-7 cells with 10 nM estradiol for 2 h causes an increase in the level of cytokeratins associated with DNA, while no increase is evident in intermediate filament-assembled or total cellular cytokeratins.

Reportable Outcomes:

Employment/research opportunities and post-doctoral research funding have not yet been presented since I have only recently begun looking for a post-doctoral position. However, the following are publications generated in the 2001-2002 award year:

1. Spencer, V. A. and Davie, J. R. (2002). Dynamic Histone Acetylation and its Role in Transcription. **Gene Ther. Mol. Biol.,** in press.
2. Spencer, V. A. and Davie, J. R. (2002). Isolation of Proteins Cross-Linked to DNA by Cisplatin. In The Protein Protocols Handbook, J. .M. Walker, ed. (Totowa: Humana Press), pp. 747-757.
3. Spencer, V. A. and Davie, J. R. (2002). Isolation of Proteins Cross-Linked to DNA by Formaldehyde. In The Protein Protocols Handbook, J. .M. Walker, ed. (Totowa: Humana Press), pp. 753-757.
4. K. L. Dunn, H. Zhao, V. A. Spencer, J. R. Davie. (2002). CTCF action at insulators. Biochem. Cell Biol. 80, 389.

Conclusions:

The presence of acetylated H3 and H4 along the PS2 gene in response to estradiol treatment suggests that H3 and H4 acetylation are involved in transcriptional elongation, as well as transcriptional initiation. In support of this, a 60-kilodalton subunit of the elongator/RNAPII holoenzyme (Elp3) is a histone acetyltransferase able to acetylate the core histones *in vitro* [10]. As well, the p300 histone acetyltransferase interacts specifically with the transcriptional initiation form of RNA polymerase II, while the PCAF acetyltransferase interacts with the transcriptional elongation form of RNA polymerase II [11].

Exposure to estrogen for 2 h increases the levels of cytokeratins associated with DNA, suggesting that exposure to estrogen promotes the interactions between cytokeratins and DNA. These interactions are most likely important for early stages of estrogen action (i.e. after 2 h exposure to estradiol). The cytokeratin 19 gene has an estrogen-response element, and the transcription of this gene is induced after 3 h exposure to estrogen [12]. However, the accumulation of cytokeratin 19 occurs after 8 h exposure [12]. Therefore, in addition to directly altering the interactions between DNA and cytokeratins, estrogen may still promote an increase in the association of cytokeratins with DNA simply by increasing the total cellular levels of cytokeratins. The cytoskeleton is linked to the nuclear matrix, the site of transcription [2]. Therefore, changes in the components of the cytoskeleton structure most likely affect gene expression by altering the organization of the nuclear matrix and DNA. In support of this, treatment of fully differentiated mouse mammary epithelial cells with cytochalasin D, an actin microfilament inhibitor, causes cells to round up and inhibits total and casein protein synthesis in a concentration-dependent manner [13]. A loss of chromatin

organization correlates with malignancy [14]. Therefore, alterations in factors that directly or indirectly affect chromatin organization most likely affect breast cancer development and proliferation. Overall, the findings of our studies suggest that exposure to estradiol causes immediate changes in both the structure of estrogen-responsive genes, and the cytoskeletal component of the tissue matrix system. Future studies will confirm our current findings of the effect of estrogen on cytokeratin and actin levels, and determine if the estrogen-induced transient increase in histone acetylation along the PS2 gene is dependent on the process of transcription.

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Appendix

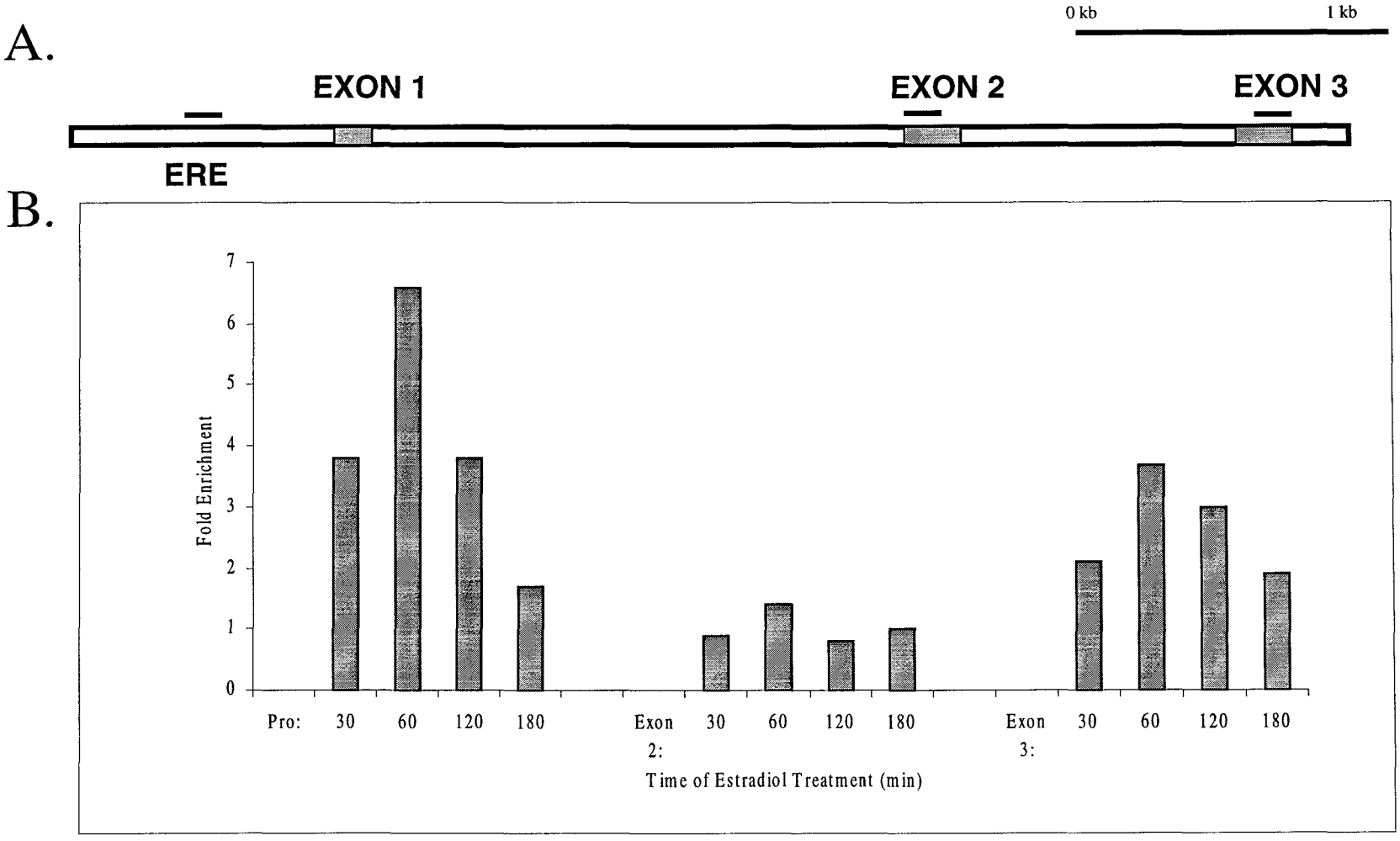


Figure 1. Acetylation of H3 along the PS2 gene in response to treatment with 10 nM estradiol for 0-180 min. (A). Schematic diagram of PS2 gene. ERE represents the estrogen response element, the region of the PS2 promoter where the estrogen receptor binds (B). Fold enrichment of acetylated H3 along the promoter (pro), exon 2 and exon 3 region of the PS2 gene.

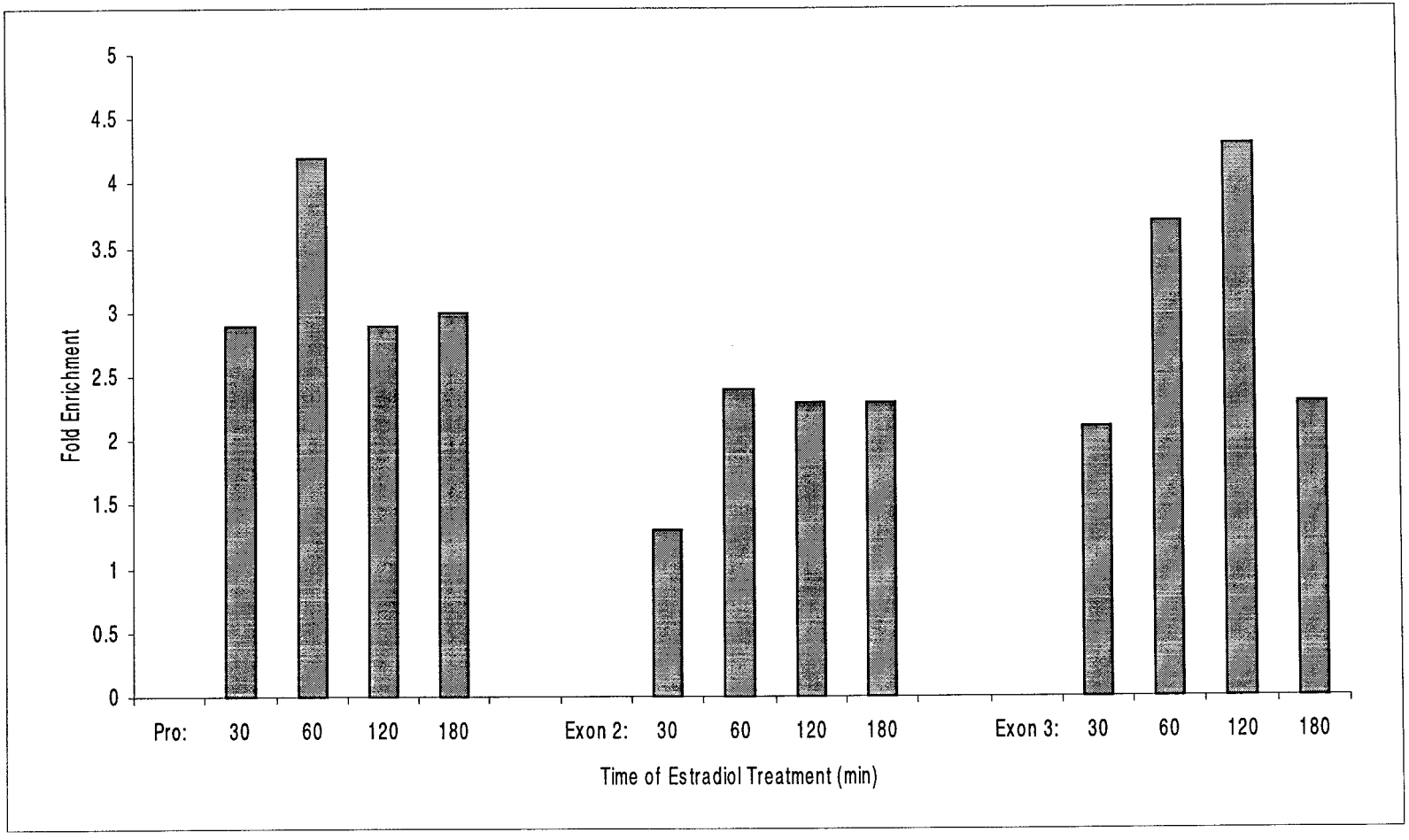
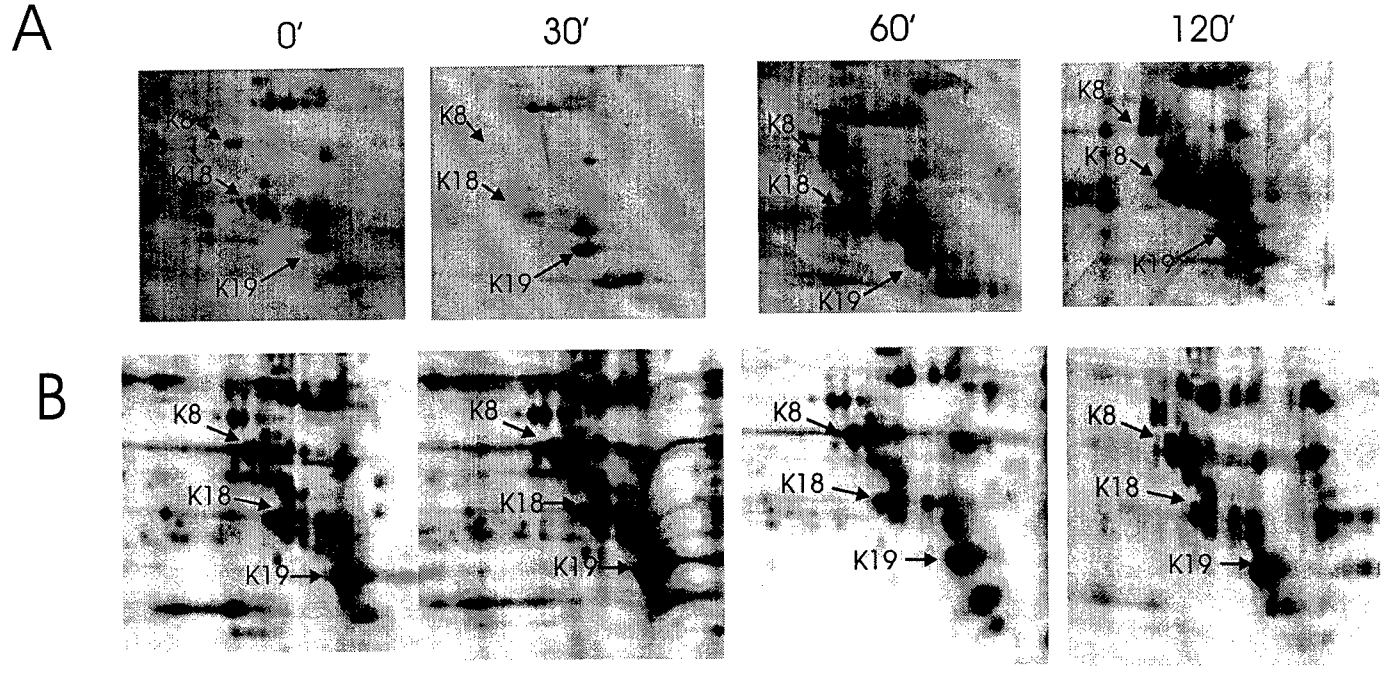


Figure 2. Fold enrichment of acetylated H4 along the PS2 promoter (pro), exon 2 and exon 3 gene regions in response to treatment with 10 nM estradiol for 0-180 min.



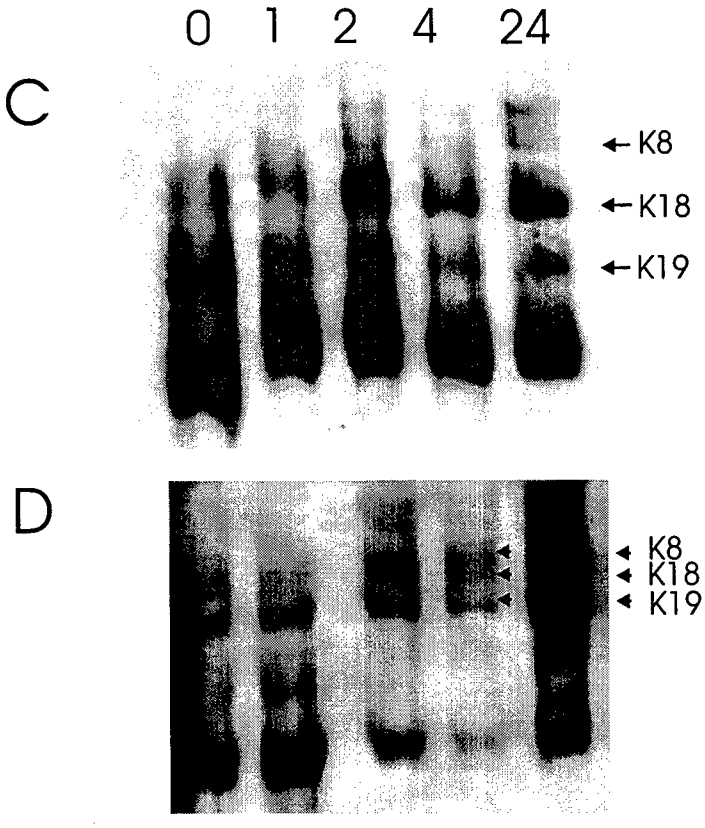


Figure 3. Treatment with estradiol increases the level of DNA-associated cytokeratins in MCF-7 human breast cancer cells. K8, K18, K19 and E2 represent cytokeratins 8, 18, 19, and estrogen, respectively. (A).Two-dimensional profile of silver-stained cisplatin DNA-cross-linked protein (20 ug) in MCF-7cells treated with 10 nM estradiol for 0 - 120 min. (B). Repeat of (A) except on a different passage of MCF-7 cells. (C). Western Blot analysis of 10 ug of cisplatin DNA cross-linked proteins from MCF-7 cells treated with 10 nM estradiol for 0 - 24 h using an antibody to cytokeratins 8, 18 and 19.(D). Repeat of (C) except on a different passage of MCF-7 cells.

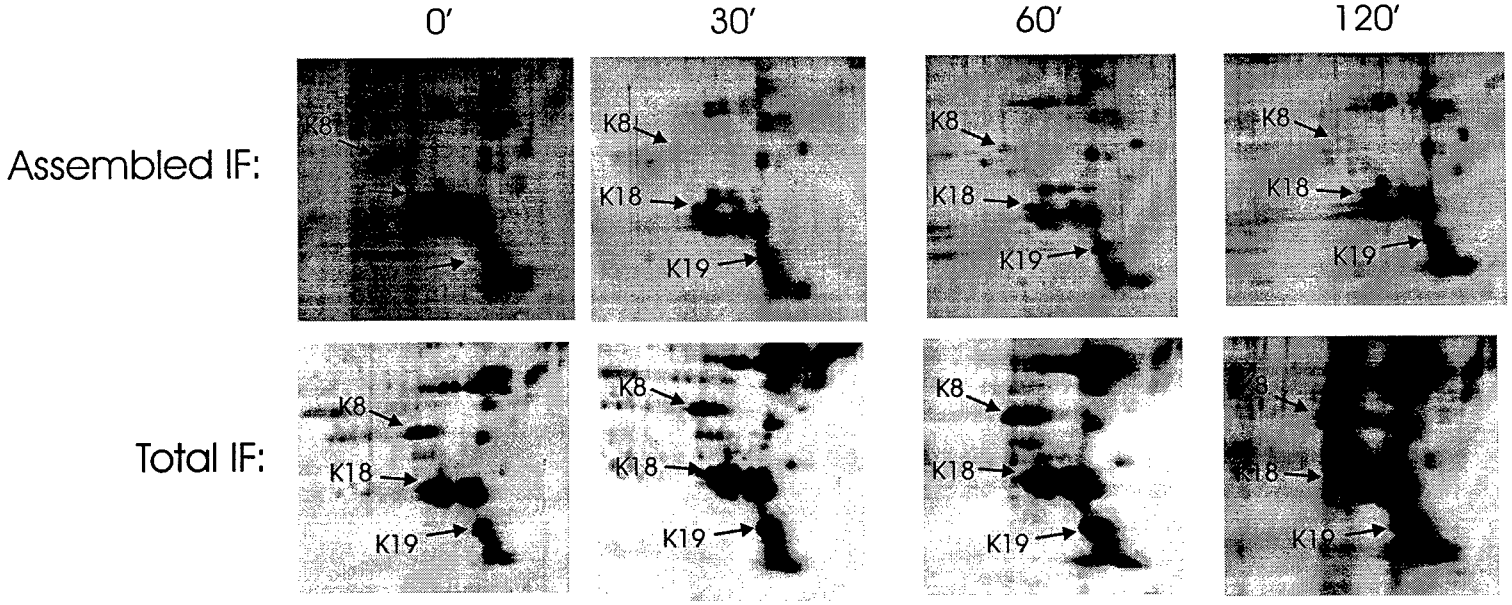


Figure 4. Estradiol does not alter the levels of assembled or total intermediate filaments in MCF-7 human breast cancer cells. MCF-7 cells were treated with 10 nM estradiol for 0 - 120 min and the total (20 ug) and intermediate filament-assembled (15 ug) cytokeratins were isolated, and analysed by two dimension gel electrophoresis folllowed by silver staining. IF, E2, K8, K18 and K19 represent intermediate filaments, estrogen, cytokeratin 8,cytokeratin 18 and cytokeratin 19, respectively.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
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